plasmid transfection-based production system (FIG. **1**). We found that a constant pH, automatically provided in commercially-available bioreactors, if used during the transfection may markedly decrease transfection efficiency (GB14/17042.7 and PCT.US2015.46927). Since then we have found other counter-intuitive ways to improve PEI-mediated transfection.

[0010] We have optimized PEI mediated transfection by finding several new approaches to constructing the plasmid DNA and PEI complex. Our research has revealed that several experimental variables are results-critical. These results-critical variables include mixing time, incubation time, DNA concentration and pH control. Our findings are surprising because the art does not teach, nor even imply, that any of these variables is significant in large-scale PEI-mediated transfection in limited volume.

BRIEF DESCRIPTION OF THE FIGURES

[0011] FIG. **1** is a flow-chart of the process steps for using PEI in a novel fixed-bed bioreactor, the iCELLis™ bioreactor.

[0012] FIG. **2** is a flow-chart of the process steps for media exchange in a novel fixed-bed bioreactor, the iCELLis™ bioreactor.

[0013] FIG. **3** shows results from our initial testing of PEI-mediated transfection only by using one plasmid and PEIPro™ transfection reagent in flasks by following the manufacturer's instructions, in an iCELLis™ bioreactor.

[0014] FIG. **4** shows the DNA concentration in our transfection mix (i.e., the plasmid DNA mixed with PEI, before adding that mixture to the host cells).

[0015] FIG. **5** shows the effect of prolonged incubation (by mixing) on DNA-plasmid particle size.

DESCRIPTION

[0016] The manufacturer of PEIPRO™ (Polyplus transfection) recommends the use of PEI at 1-6 μl of PEIPRO™ per 1 μg of DNA for HEK293 cells. For adherent cells, the recommended amount of DNA is 0.1-0.58 μg/cm2, depending of the type of the flask when the total concentration is up to 0.029 μg/μl (Polyplus, PEIpro in vitro DNA transfection reagent protocol).

[0017] First we did "as instructed" by the art in a small scale. A problem came when we tried to scale that up, however, because we realized that the art-recommended approach does not work in a large scale where the working volume is limited. We initially tested PEI mediated transfection only by using one plasmid and PEIPRO™ transfection reagent in flasks by following the manufacturer s instructions (FIG. **3**). We used a DNA amount from 100 to 400 ng/cm^2, evaluated using a DNA:PEI ratio of 1:1. Results showed that 300-400 ng/cm^2 total DNA concentration achieved the highest transfection efficiency (up to 98% positive cells) in our cells. The DNA concentration in our transfection mix (i.e., the plasmid DNA mixed with PEI, before adding that mixture to the host cells) we evaluates was up to 15 μg/ml. Also, we used different PEI ratios, and found that the PEI ratio has an effect on the transfection efficacy (FIG. **4**). We found that the best transfection efficacy was achieved by using PEI:DNA ratio of about 1:1.5.

[0018] In our next experiments, the total DNA concentrations per cm^2 were the same, but we used DNA which contained four different plasmids, as is typically used for retroviral vector production. Virus production by producer cells which have been transfected with several plasmids is tricky because the producer cells require a larger volume of plasmid DNA (i.e., several different plasmid constructs) than a typical recombinant protein production where only one plasmid is used to express the one polypeptide of interest. We found that the highest titers were achieved using the best conditions shown in a previous experiments.

[0019] The first PEI mediated plasmid transfection in an iCELLis™ bioreactor was done by Lennaertz et al. when they produced AAV in a 0.53 m^2 fixed-bed bioreactor. Their results showed that plasmid transfection is feasible in the low bed height laboratory-scale iCELLis® nano bioreactor (Lennaertz et al., 2013).

[0020] Our next aim was to test virus production using iCELLis™ fixed bed bioreactor with the same conditions than in flasks (manufacturer's instructions) but this time using the largest 4 m^2 iCELLis™ Nano bioreactor (fixed-bed comparable for 500 m^2 in iCELLis™ 500) (FIG. **1**). It was surprisingly observed that actually the recommended transfection conditions are not scalable and applicable for iCELLis™ bioreactors, especially in higher bed height (>2 cm) bioreactors due to its limited working volume for high total cell number if the DNA amount would have been kept the same per cell or per cm^2. In other words, if the same plasmid transfection mix would have been used, it would not fit into the bioreactor or would have required full medium exchange during the transfection. The iCELLis™ Nano is a small scale equipment where the full medium exchange can be done fast and is not limiting step in a process. In contrast, the iCELLis™ 500 at scale, the full medium exchange is not practical process step because it takes time and may be influencing on cell viability due to the fact that during the draining, stirring is closed and the cells on the upper carriers are without the medium. Thus there was a need to decrease the volume in transfection mix which lead to higher DNA (plasmid) concentration in a mix but transfection efficacy was decreased (Run 1, table 1). We were able to avoid formation of aggregation by continuous mixing of the DNA-PEI mix before adding to the cells or with shorter incubation time (table 1). If we would have kept the DNA concentration the same in a iCELLis nano than in flasks, total of 800 ml transfection mix would have needed which is the maximal working volume in the iCELLis nano scale. Alternatively, recirculation is required in iCELLis fixed bed bioreactor when the ≥4 cm bed height is used. In 2 cm fixed bed, lower cell amount do not require that much plasmids (transfection mix) and thus the bioreactor volume is not limiting factor. Thus Lennaertzt et all. did not face any problems in their transfection (Lennaertz et al 2013). We tested the transfection by making plasmid-PEI mix in a large volume (lower concentration), and adding it to the bioreactor but because the bioreactor working volume was exceeded, we used a re-circulation loop (FIG. **2**) (run 7, table 1).

[0021] Another surprise was seen during the large scale transfection. After adding transfection mix into the bioreactor, everything seem to be normal but when sampling the bioreactor, chemical reaction was observed when normal shape plasmid tube "collapsed" or "melted" because of the medium sample with transfection mix. It was find out that PEI could react with CO_2. Based on all what happened and what was seen, it was concluded that CO_2 flow (pH control)